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Responsive, Water Soluble Europium(III) Luminescence Probes

Sergey Shuvaev*, Matthieu Starck and David Parker *

Department of Chemistry, Durham University, South Road, Durham DH1 3LE, UK.

E-mail: david.parker@dur.ac.uk; sergey.shuvaev@dur.ac.uk

Abstract: The design principles, mechanism of action and performance of europium(III) complexes that serve as strongly emissive and responsive molecular probes in water are critically discussed. Examples of systems designed to assess pH, selected metal ions and anions, including chiral species, as well as selected small molecules and biopolymers are considered, and prospects evaluated for improved performance in more complex biological media such as in bio-fluids and within living cells. Modulation of the emission spectral form, lifetime and degree of circular polarisation can be used to quantify the spectral response and permit calibration.

Introduction

The genesis of lanthanide bio-imaging probes can be traced to two discoveries: the observation of the red emission of europium by Crookes in 1885 and the report of its sensitised emission made by *Weissman* in 1942^[1]. Each discovery epitomises the advantageous optical features of lanthanide complexes – narrow bands in emission with energies only slightly affected by the nature of the ligand, and a large separation (a “pseudo-Stokes’ shift”) between the absorbing (ligand-antenna) and emitting (lanthanide ion) photon energies. These favourable optical properties create a myriad of opportunities to devise responsive systems by judicious ligand design. Europium(III) complexes have particular advantages over other lanthanide complexes in biologically relevant applications: the higher transparency of biological tissue towards the red region of the visible spectrum; their high inherent brightness and low sensitivity to quenching by oxygen; the presence of three excited states (5D_2 , 5D_1 and 5D_0) spanning the range 17500-21000 cm^{-1} that are lower in energy than the triplet levels of many different types of ligands; the presence of an environmentally insensitive magnetic-dipole transition 5D_0 to 7F_1 in the luminescence spectrum, (Figure 1), that can be used as an internal reference to the hypersensitive transition bands around 620 (5D_0 - 7F_2) and 700 nm (5D_0 to 7F_4), and the high emission anisotropy factors, g_{em} , in circularly polarised luminescence (CPL) spectra.

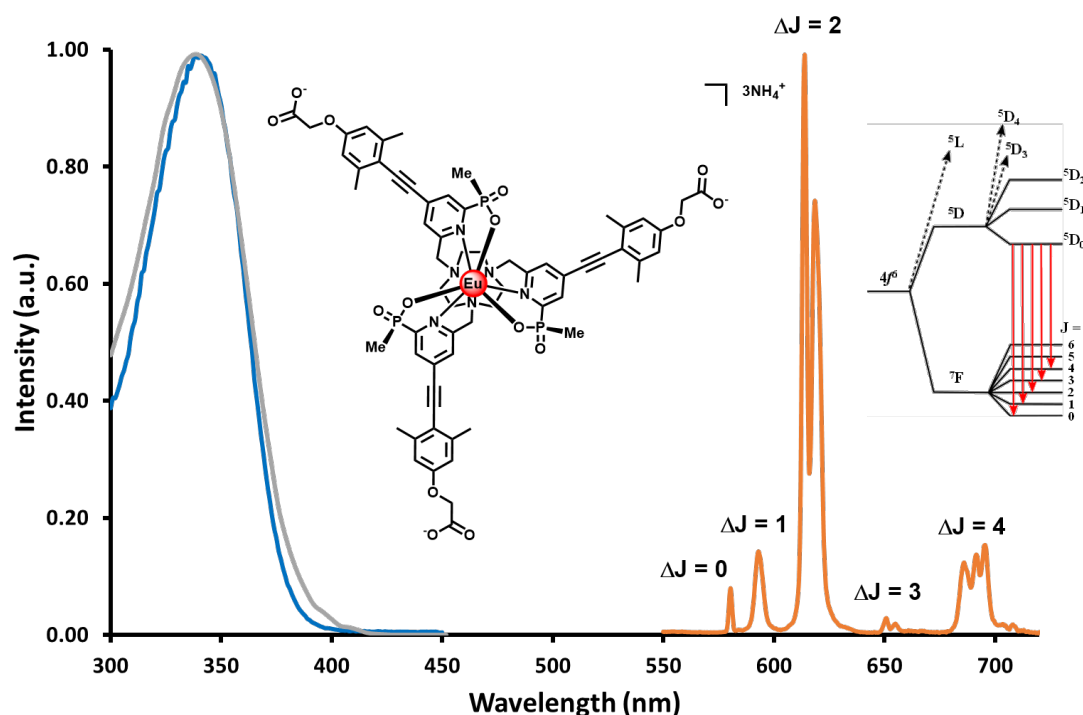


Fig. 1 A typical Eu(III) emission spectrum (right) of a nine-coordinate complex (H_2O , 295K), showing absorption/excitation spectra (left).

A simplified photophysical schematic pathway, involving energy transfer from an absorbing antenna with appropriate singlet (S) and triplet (T) energies in the sequence, $\text{S} \rightarrow \text{T} \rightarrow \text{Eu}^{3+}$, has been shown to be valid for the majority of complexes studied. There are a few cases where the pathway involves competitive direct energy transfer from the singlet excited state to the lanthanide ion, but these are generally considered to be rare. [2][3] The photophysical phenomena observed in certain special cases requires further consideration. For example, a significant dependence of emission and excitation spectra on solvent polarity can be observed sometimes, and a very low efficiency of Eu^{3+} sensitisation has been reported in certain systems, notwithstanding an optimal value of the energy gap between triplet level and a resonance energy level of Eu^{3+} . The presence of donor and acceptor groups within the same molecule, separated by a conjugated system, gives rise to 'charge transfer' states. The term can be used in a broad definition to embrace all energy states that involve at least partial separation of charge in an excited state. Accordingly, the energy of these transitions can be highly dependent on the polarity of the solvent, and therefore the observation of solvatochromism provides evidence in support of the assignment of these bands as charge transfer (CT) transitions.

The question of the participation of these bands in the sensitisation pathway of Eu^{3+} emission has been often raised. It has been suggested that the intervention of an intra-ligand charge-transfer (ILCT) state can efficiently compete with a pathway involving the ligand triplet, for sensitisation of Eu^{3+} luminescence, provided that it has a suitable energy to match the resonance levels of Eu^{3+} . Evidence in favour of the predominant singlet character of these bands is given by observation of direct excitation into the ILCT band, with a decay rate of the

order of nanoseconds and the absence of triplet oxygen quenching of metal emission.

Besides ligand-centred ILCT states, ligand-to-metal charge transfer (LMCT) states, involving intermediate reduction of Eu^{3+} to Eu^{2+} can be observed in the absorption/excitation spectra of europium(III) complexes. The reduction potential for $\text{Eu}^{3+}/\text{Eu}^{2+}$ is the lowest in the lanthanide series, e.g. -0.35 V for the aqua ion, and about -1.1 V for Eu(III) complexes with many strongly coordinating octadentate ligands that stabilise Eu(III) with respect to Eu(II) [4],[5]. The energy of these broad LMCT bands, with widths in the range 5000-15000 cm^{-1} , varies considerably depending on the electron-donating ability of the ligand[6]. The states have mainly singlet character. If an LMCT state is located close to the resonant excited state levels of Eu^{3+} , it serves as an efficient quenching pathway, permitting non-radiative depopulation of an excited state of Eu^{3+} . Since the energy of the LMCT state is proportional to the reduction potential of the triply charged lanthanide ion, isostructural complexes with lanthanide ions other than Eu^{3+} should shift this band hypsochromically in their absorption spectra, by a fixed value that is independent of the nature of the ligand.

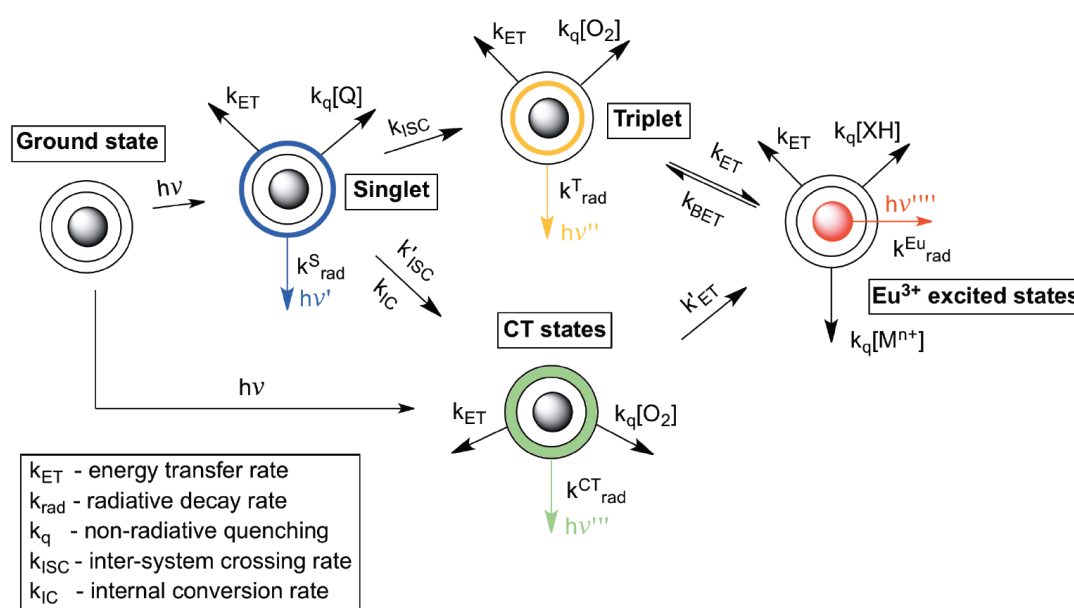


Fig. 2 Schematic representation of the major energy transfer processes occurring in responsive europium(III) complexes, including interactions with quenching species (Q), dioxygen, proximate vibrational oscillators (X-H; X = C, N, O) and transition metal ions (M^{n+}).

A variety of excited state pathways characterises the sensitisation of Eu^{3+} emission (Fig. 2). External perturbation of these energy-transfer processes allows different types of luminescent responsive probes to be devised. These probes can be divided into three major groups: those involving a change in the overall intensity of Eu^{3+} -centred emission; those involving changes of the spectral profile without major change of total emission intensity; those that involve changes in *both* the overall intensity and the emission spectral signature.

The first group can be subdivided into two sections, depending on the multiplicity of the excited state involved.

Perturbation of the singlet excited state. In this case, efficient energy transfer may occur between the singlet state of the antenna and the singlet state of another molecule (or moiety within the same molecule), either by a long-range (Förster mechanism) or by a close contact interaction (e.g., π - π stacking). Alternatively, the coupling to a charge transfer state (ILCT or LMCT) can lead to deactivation of the antenna S_1 state. In the limit, this process may involve complete electron transfer, and is often simply termed as photoinduced electron transfer.

Perturbation of the triplet excited state. Deactivation of the triplet level can occur either by back energy transfer from resonant levels of Eu^{3+} , if the energy gap is less than *ca.* 1800 cm^{-1} , or by ligand phosphorescence or non-radiative vibrational quenching, if the energy gap is too large and the rate of energy transfer is relatively slow. Such back energy transfer processes are much more common in analogous terbium complexes, owing to its higher lying $^5\text{D}_4$ excited state. Alternatively, coupling with a charge transfer state (ILCT or MLCT) can also quench a triplet state. An efficient deactivation by energy transfer to triplet oxygen in solution is occasionally observed, leading to formation of singlet oxygen.

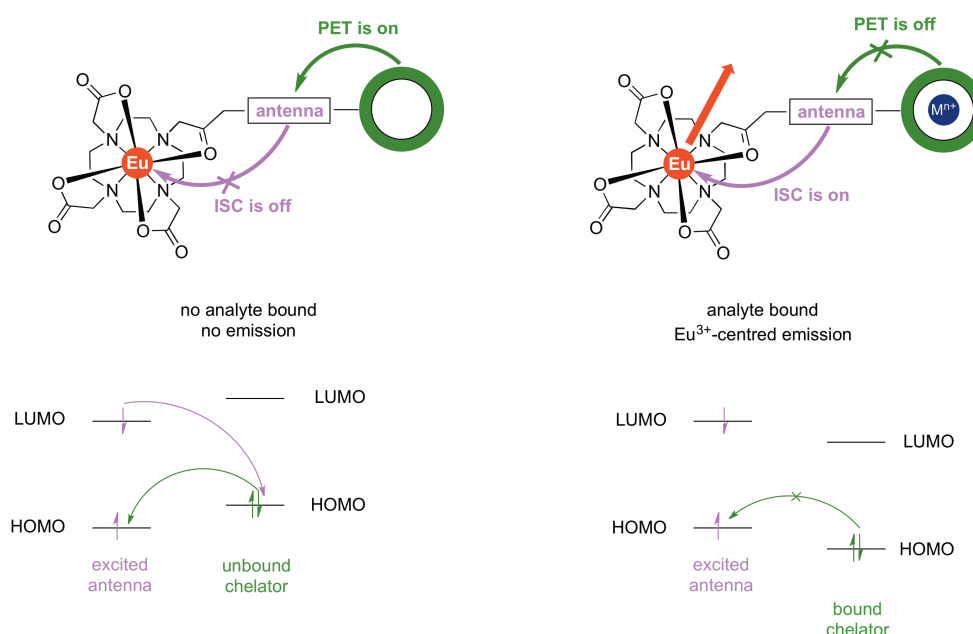
Perturbation of the excited states of Eu^{3+} Non-radiative deactivation of the excited state of the europium ion can occur by energy transfer to electronic energy levels of transition metal ions (e.g. Cu(II) , Mn(II) or Co(II)), or by a FRET-mechanism to different acceptor molecules/ions. These probes can be ratiometric in nature and may be addressed using excitation spectroscopy, if the induced response to an external stimulus is accompanied by an absorption spectral shift.

The second group involve changes in the coordination environment of the Eu^{3+} ion. They are usually ratiometric probes and typically involve observation of the 'hypersensitive' electric-dipole transitions whose intensities can be referenced to the emission intensity of the magnetic-dipole transition, $^5\text{D}_0$ - $^7\text{F}_1$ that is usually insensitive to change of the primary coordination sphere. Typically, perturbation of the coordination environment is accompanied by variation in the number of quenching oscillators X-H (X = O, N, C). Such a change will alter the overall intensity as well, if the conditions for favourable Franck-Condon overlap are met. The third group includes probes that combine different sensing mechanisms, illustrated by the probes described in the two previous sections.

Several recent reviews^{[7],[8],[9]} have addressed advances in the development of lanthanide-based probes, including europium(III) complexes. Whilst providing some insight, none presents a succinct and critical study that focuses on water-soluble and bright europium(III) complexes. This topic is addressed herein, with an emphasis on exemplifying the mechanisms involved that may lead to a useful analytical response. This review is *not* comprehensive and a judicious selection of examples has been chosen to emphasise differing mechanistic issues.

Photoinduced electron transfer (PET)

Quenching involving partial or complete electron transfer, often referred to as PET, is the mechanism providing response towards external stimuli in the majority of luminescence probes created. In its simplest form, the PET quenching mechanism in Eu^{3+} complexes can be rationalised, using the example of metal-sensing probes, (Scheme 1). The HOMO level of the metal-binding unit, when the metal ion is not bound, should lie above the HOMO level of the antenna that sensitises the europium-centred emission to quench its excited state. When the binding moiety chelates the metal ion, its HOMO may become lower in energy than the HOMO of the antenna, thereby eliminating quenching and promoting sensitisation of Eu^{3+} luminescence. The correct match between frontier orbitals in the chelating moiety and in an antenna, before and after the binding event, determines the magnitude of the 'turn-on' enhancement and hence the detection limit. The energetics of these processes are readily calculated using redox potentials and excited state energies, via the Rehm-Weller equation, as noted in earlier reviews. [10]



Scheme 1 Schematic mechanism of PET quenching in a Eu^{3+} complex in a hypothetical example of a metal-sensing probe.

Metal ion sensing

Transition metals play an essential role in many biological processes occurring within a living cell, and therefore bio-imaging probes that combine high sensitivity and selectivity towards these metal ions are highly desirable. Unfortunately, there is still some inconsistency regarding the desired sensitivity of these probes, given the fact that only the metal ions in a kinetically labile form can be detected. Moreover, the local metal ion concentration varies considerably with the nature of the cell compartment and the type of cell in question.

In the past few years, several papers dealing with europium complexes designed for sensing of transition metals have been published, in particular Zn^{2+} , Cu^{2+} , K^{+} and Hg^{2+} ions. The usual mechanism behind the analytical response in these

systems is based on an efficient quenching of Eu^{3+} emission due to energy transfer between higher energy states of europium, the excited state of the ligand and the d-orbitals of metal ions. However, it was recently shown that paramagnetic Cu^{2+} complexes may actually increase the quantum yield of europium emission, by quenching the ILCT state, and thereby increase the probability of inter-system crossing to the triplet state of the ligand. [11]

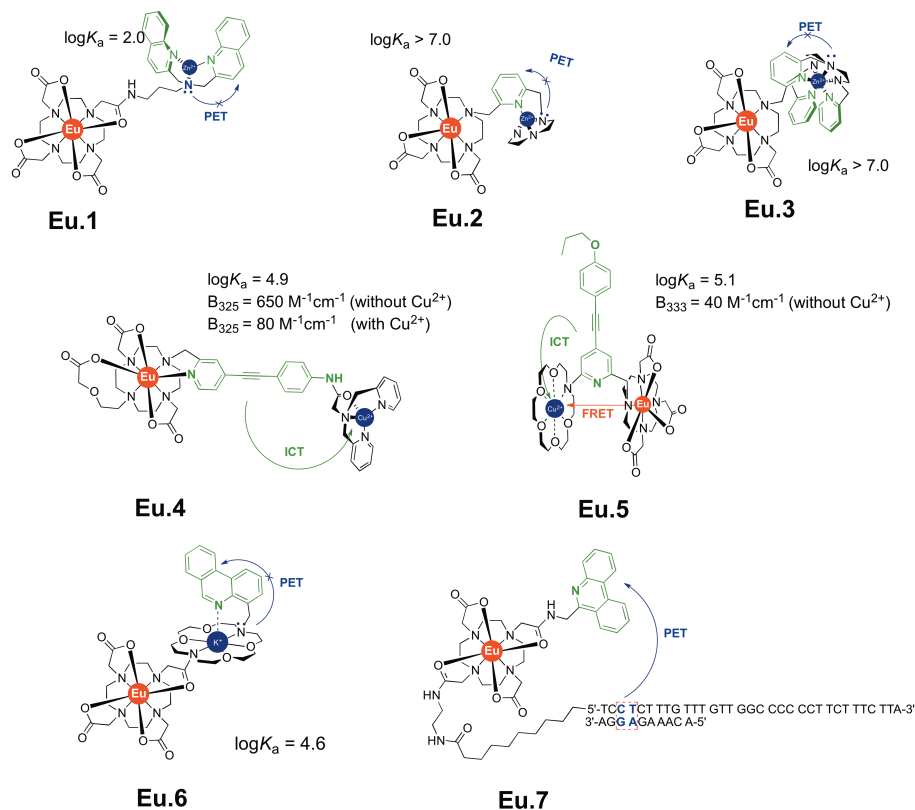


Fig. 3 Molecular structures of metal-sensing probes. Moieties involved in sensitisation are depicted in green, whilst arrows indicate plausible mechanisms involved in Eu^{3+} quenching. Binding constants, K_a , and probe brightness at the excitation wavelength, $B_\lambda = \epsilon\phi_{\text{em}}$, are depicted where available. ICT = internal charge transfer; PET = photoinduced electron transfer; FRET = Förster resonance energy transfer.

The selectivity of metal-sensitive bio-imaging probes is provided by the integration of a binding moiety that is chemoselective towards the desired metal ion (Figure 3). In the most useful systems, the emission spectrum of the europium ion changes upon binding, and the corresponding change in the relative emission intensity can be plotted as a function of metal ion concentration.

Zinc ions play a significant role in intra- and intermolecular signalling when present as a kinetically labile aqua ion species. [12] Although the concentrations of Zn^{2+} in the cytosol of human cells usually lie in the picomolar range, its free concentration in organelles can be several orders of magnitude higher, reaching the micromolar range. [13] To bind Zn^{2+} ions selectively, derivatives of tetradentate N_4 tripodal ligands are often employed, which have a high affinity towards both Zn^{2+} and Cu^{2+} ions. For example, for tris-(2-pyridylmethyl)amine (TPA) ($\log K = 11.0$; $K = [\text{ML}]/[\text{M}][\text{L}]$), and for Cu^{2+} binding the value of $\log K = 16.2$. [14] The higher affinity towards Cu^{2+} over Zn^{2+} , together

with an efficient quenching of europium emission by the former ion, inevitably leads to a very poor performance of these 'Zn²⁺-selective probes' in the presence of even low concentrations of free Cu²⁺. The recently reported Zn²⁺ probe **[Eu.1]**^[15], represents a further development of the TPA system, and has been attached to a [Eu.DOTA] core. Unsurprisingly, it showed high selectivity towards Zn²⁺ in the presence of Na⁺, K⁺, Ca²⁺ and Mg²⁺. The conformational changes in the position of the pyridine (or quinoline) moieties upon zinc binding increase the emission intensity of the complex, whilst addition of Cu²⁺ drastically quenches the luminescence.

An attempt to design a more selective zinc probe was made by *Tripier*, based on a 9-N₃ core as a binding site for Zn²⁺.^[16] This Zn-binding site tends to favour a distorted tetrahedral coordination environment, disfavours the binding of square-planar copper (II), in the case of **[Eu.2]** and **[Eu.3]**^[16]. No change in the Eu hydration state occurred on zinc binding, and only **[Eu.2]** showed a variation in the $\Delta J = 2/\Delta J = 1$ emission intensity ratio.

On the other hand, the high affinity of TPA-derivatives towards Cu²⁺ can be employed to create Cu²⁺-selective probes, as demonstrated by *Wong*. A Cu²⁺-selective probe **[Eu.4]** was studied, employing a DOTA platform with a dipicolylamine moiety that was used for selective binding of Cu²⁺. Efficient quenching of luminescence was observed in the presence of Cu²⁺.^[17] The emission signal was recovered, once the complex was exposed to H₂S solution, resulting in CuS precipitation. High selectivity over alkali and transition metals was observed. However, this approach involving formation of insoluble copper (II) sulphide is obviously inapplicable to cellular studies, as it would likely cause cell apoptosis. Moreover, an efficient quenching of luminescence signal upon Cu²⁺ binding is barely useful for cellular imaging. The same group suggested a more complicated approach^[18] to detect Cu²⁺ with concomitant detection of H₂S. They devised the complex **[Eu.5]** using an aza-18-crown-6 ligand attached to an alkynyl-pyridine moiety; selective binding of Cu²⁺ was observed, efficiently quenching europium emission. Upon addition of H₂S, a drastic increase in the emission intensity was observed that exceeded that of the initial europium complex, without bound copper. This observation was tentatively attributed to the formation of a new complex with Na₂S bound to both Eu³⁺ and Cu²⁺. However the same flaws, including the large decrease of the emission intensity upon binding the Cu²⁺, will inhibit biological application. The macrocyclic N₂O₄ binding moiety – diaza-18-crown-6- well known for its relatively high affinity towards K⁺ ions – was employed by *Pierre* in the design of a selective potassium luminescent probe, **[Eu.6]**^[19]. The magnitude of the binding constant suggested that the nitrogen atom of the phenanthridine moiety participates actively in binding to K⁺, aiding direct detection in extracellular media.

A more elaborate, yet more selective approach is based on the use of aptamers – single stranded nucleotides that have a high affinity and selectivity towards a certain target, e.g. metal ion, protein or small molecule. *Pierre* designed a cyclen-based europium complex **[Eu.7]** with an attached Hg²⁺-selective aptamer and a phenanthridine-derived chromophore^[20]. The excited state of the latter antenna is efficiently quenched by charge transfer via π - π stacking interactions with purines, but not with pyrimidines. Therefore, in the absence of Hg²⁺ ions the chromophore intercalated the dsDNA, whose purine bases efficiently quench the

Eu³⁺-centred emission. However, when Hg²⁺ ions are added the aptamer releases a short complementary strand and the phenanthridine moiety de-intercalates dsDNA, leading to a large increase of the Eu³⁺ emission intensity. The detection of Hg²⁺ ions on a micromolar level was observed with high selectivity over other transition metal ions. However, this system is not ratiometric in nature, so that difficulties in calibration will limit utility.

Sensing of biologically relevant anions

The design of anion-selective probes is governed by the physicochemical properties of the analyte. Key properties to consider include the nature of anion binding to the metal centre, the potential to engage in stabilising hydrogen bonding or π - π -stacking with the probe, the anion pK_a and its charge density. Such considerations have been taken into account in the series of ratiometric Eu(III) probes that have been devised for citrate, lactate, urate and bicarbonate, that have been used successfully in complex media and applied in biological studies both *in vitro* and *in cellulo*.^{[21],[22],[23]}

Europium complexes with high selectivity towards specific anions are still relatively scarce. Here, the discussion is restricted to two limiting cases – examining fluoride and nucleotide sensors. In the first case, the small fluoride ion can replace a water molecule in the coordination sphere of Eu³⁺ at sub-millimolar concentrations, if additional stabilization through formation of hydrogen bonds with F⁻ is provided. In the second case, the bulky nucleotide molecules possess both a nitrogenous base capable of π - π -interactions with a probe and a phosphate group which can coordinate to Eu³⁺.

In the majority of cases discussed below, (Figure 4) the binding molecule/ion replaces a coordinated water molecule(s), so that the photophysical response accompanying the binding event is an increase of total luminescence intensity, upon removal of the O-H quenching oscillators. In parallel, alterations to the Eu³⁺ spectral profile occur, caused by changes in the nature and polarisability of the coordinated donors that define the ligand field.

Fluoride ions play an essential role in many physiological processes, such as bone and enamel mineralization. However, if fluoride is present in excessive amounts, it can lead to disturbances of bone homeostasis (skeletal fluorosis) and enamel development (dental fluorosis)^[24]. Therefore, its concentration in drinking water and biological tissues needs to be monitored. The fluoride anion is comparable in size with OH⁻ (1.29 Å for F⁻ and 1.32 Å for OH⁻), and their competitive binding can present a challenge. However, within the physiologically relevant pH range, the concentration of OH⁻ in aqueous solutions is very low and does not usually hamper F⁻ binding. As a hard Lewis base, fluoride has a relatively high affinity towards hard Lewis acids, such as lanthanide ions. Therefore, the use of lanthanide complexes as fluoride probes is sensible. However, the usually observed binding constants of fluoride ions for lanthanide complexes in water are relatively low ($\log K_a = 1.5-3$), due to the high free energy of hydration of the fluoride ion ($-\Delta G^\circ_{\text{hydr}} = 465 \text{ kJ/mol}$). Efficient binding has been observed only with cationic complexes where a water molecule in the ninth coordination site of europium can be replaced by the fluoride ion, stabilized by additional interactions. For instance, the coordinated F⁻ can be additionally stabilized by F⁻---CH interactions, as in the europium complex reported by

Butler^[25]. The europium complex **[Eu.8]** based on a cyclen ring and bearing two quinoline chromophores in *trans* positions has a coordinated water molecule that can be replaced by F⁻, giving rise to a 9-fold increase of luminescence intensity. In parallel, a large change of the emission profile was observed within the relevant range (20-210 μM), typical for water fluorination. Although no anion binding was observed in the case of Cl⁻, Br⁻, I⁻, NO₃⁻, HSO₄⁻, HPO₄²⁻, CH₃CO₂⁻, competitive binding of HCO₃⁻ was revealed, resulting in similar photophysical changes. The high selectivity towards F⁻ and high stability of the formed complex ($\log K_a = 4.1$) was attributed to an additional stabilization through formation of relatively strong C-H...F...H-C interactions between coordinated F⁻ and quinoline moieties.

Another example, where stabilization of fluoride binding by hydrogen bond formation was reported by Tripier^[26]. In this case, a fluoride anion bridges two cationic cyclen-based europium complexes bearing two picolyl and two acetamide pendant arms featuring linear Eu--F--Eu bonding array. Upon addition of fluoride, two water molecules are displaced from each site by one fluoride ion, giving rise to the 'sandwich'-like structure, **[Eu.9]**, that is stabilized by π - π stacking interactions between opposite pyridyl rings. The fluoride ion gains additional stabilization inside this double-decker structure by formation of C-H...F...H-C hydrogen bonds with pyridyl moieties. When pyridyl moieties were substituted with two other acetamide arms, the formation of 'sandwich'-like structure was not observed and substantially lower affinity towards F⁻ was discovered, emphasising the importance of the pyridyl units to enhance fluoride binding.

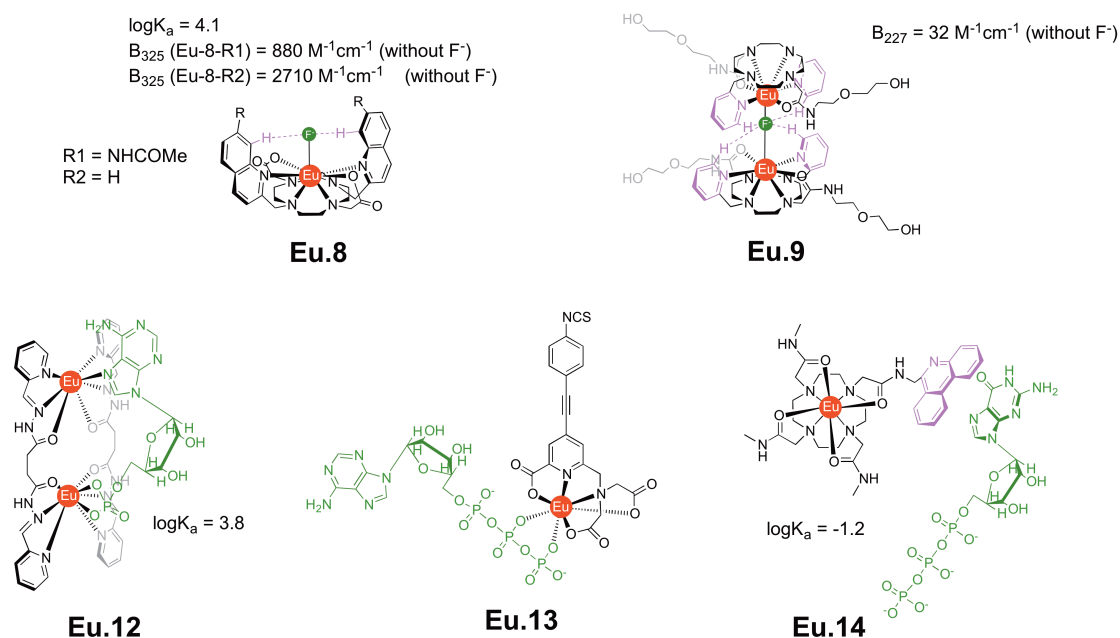


Fig. 4 Structures of selected europium complexes used for anion-sensing. The analyte is depicted in green, whilst moieties involved in stabilisation of the final aggregate are depicted in purple. **[Eu.10]** and **[Eu.11]** are analogues of **[Eu.9]** with the coordinating acetamide groups replaced by carboxylates and the pyridyl moieties substituted by indazoles, respectively.

In a subsequent study,^[27] Tripier modified the complex by replacing the acetamide arms with carboxylate moieties to reduce the overall complex charge,

alleviating repulsion during formation of the putative dimer. Substituted pyridyl units were replaced with indazole moieties, in order to provide additional stabilization via hydrogen bonding between F⁻ and NH groups of indazole. An increased molar extinction coefficient also resulted. The dimeric complex, **[Eu.10]**, showed very high sensitivity (24 nM) and selectivity towards fluoride anions over Cl⁻, Br⁻, HCO₃⁻, CH₃CO₂⁻ and HPO₄²⁻, resulting in the 22-fold increase of the overall emission intensity, accompanied by a pronounced change of the spectral profile. Analysis of the crystal structure reveals that the high association constant (log K_a = 13.0) can be explained by stabilisation of the dimer by four hydrogen bonds formed between the nitrogen atoms of the indazole moiety of one complex and the oxygen atoms of the carboxylate arms of another, as well as by formation of a Eu--F--Eu bond. A symmetric europium complex **[Eu.11]**³⁺ based on the cyclen core with four pyridyl substituents was analysed by *Faulkner* [28], revealing a high affinity towards F⁻ (log K_a = 5.0). Since no competitive binding studies have been carried out, the selectivity of the proposed complex towards fluoride ions remains unclear, although additional stabilisation of F⁻ might be expected *via* formation of hydrogen bonds with the nitrogen atoms of the pyridyl groups.

Adenine nucleotides – adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) – play an essential role in bioenergetics, participating in energy transfer processes required for normal functioning of the cell. In order to create luminescent europium probes that can bind one adenosine derivative selectively, steric control should be imposed by ingenious design of the ligands, so that the binding interaction involves both the adenosine and phosphate parts of the molecule. In general the affinity order, determined by the charge of the phosphate moiety, is usually found to be ATP>ADP>AMP, and a different order of selectivity between these species remains a challenge. With this in mind, *Albrecht* [29] has studied a coordinatively unsaturated double-stranded helical dinuclear europium complex **[Eu.12]** that causes a significant 64-fold increase of luminescence intensity upon binding AMP molecules with selectivity over ATP and ADP, as well as other biologically relevant ions. Two strands of *bis*(tridentate) diamide ligands encapsulate two Eu³⁺ ions, whose coordination spheres are saturated with water molecules and nitrate ions. Upon addition of AMP, bound water molecules and nitrate ions are replaced by one AMP molecule, coordinated by a nitrogen atom of the adenosine group to one Eu³⁺ ion and by two oxygen atoms of a phosphate group to another Eu³⁺, leading to an increase in the emission intensity of the complex. Computational studies suggested that an extension of the phosphate chain in ADP and ATP leads to a repulsive interaction; no significant binding was observed in those cases. Competitive binding data established the high stability of the **[Eu.12-AMP]** aggregate, consistent with formation of a triple-stranded structure.

By tuning the degree of saturation of coordination sphere of Eu³⁺, the selectivity towards different nucleotides can be varied. For instance, *Schäfferling* reported a family of europium complexes **[Eu.13]**³⁺ with an alkynylpyridine sensitizer that differ in the number of acetate arms, giving 5-, 6- and 7-dentate ligands. [30] These complexes showed quite different affinities towards AMP, ADP, ATP and cyclic AMP. The europium complex with a heptadentate complex showed no affinity towards either nucleotide, whilst the complex with the

hexadentate ligand exhibited poor selectivity. The complex with the pentadentate ligand revealed relatively good selectivity towards ATP over ADP, and a low affinity towards AMP, following the order of decreasing charge. The similar affinity of the latter complex towards ATP and polyphosphates – the by-product of hydrolysis of ATP into AMP – suggests that only the phosphate part of ATP participates in binding to the complex. The system was shown to be reversible in its binding to ATP. However, pentadentate europium complexes are usually rather unstable with respect to metal decomplexation and this may occur in solution over time or as the concentration of the coordinating anion increases.

The ability of a nitrogenous base to participate in π - π stacking interactions can be used to discriminate between different nucleotides, e.g. pyrimidines and purines, as shown by *Pierre*^[31]. A phenanthridine moiety attached to an amide arm of the cyclen-based europium complex, **[Eu.14]**, can participate in favourable π - π stacking interactions with nitrogenous base of nucleotides. This interaction quenches the excited state of the phenanthridine antenna, due to photoinduced electron transfer (PET), leading to a decrease of the total emission of Eu^{3+} . Both, the magnitude of the π - π stacking interactions, the efficacy of PET, and hence the sensitivity of the probe depends on the match between the LUMO level of the phenanthridine moiety and the HOMO of the nucleotide. The more conjugated purines have a higher lying HOMO level than related pyrimidines, and hence much lower sensitivity towards the latter was observed.

pH-sensitive probes

Deviation from standard pH values in cells can indicate malfunctioning of the cell, e.g. in lysosomes where mis-regulation of pH is linked to a wide range of lysosomal storage diseases. Therefore, to gain better insight into the processes accompanying different diseases at the cellular level, bright and preferably ratiometric molecular probes are required. To modulate a reversible response as a function of pH in europium complexes, a pH-sensitive moiety with the desired pK_a value should perturb the coordination environment of Eu^{3+} - either by a change of the electron demand at the europium ion or by varying the coordination environment.

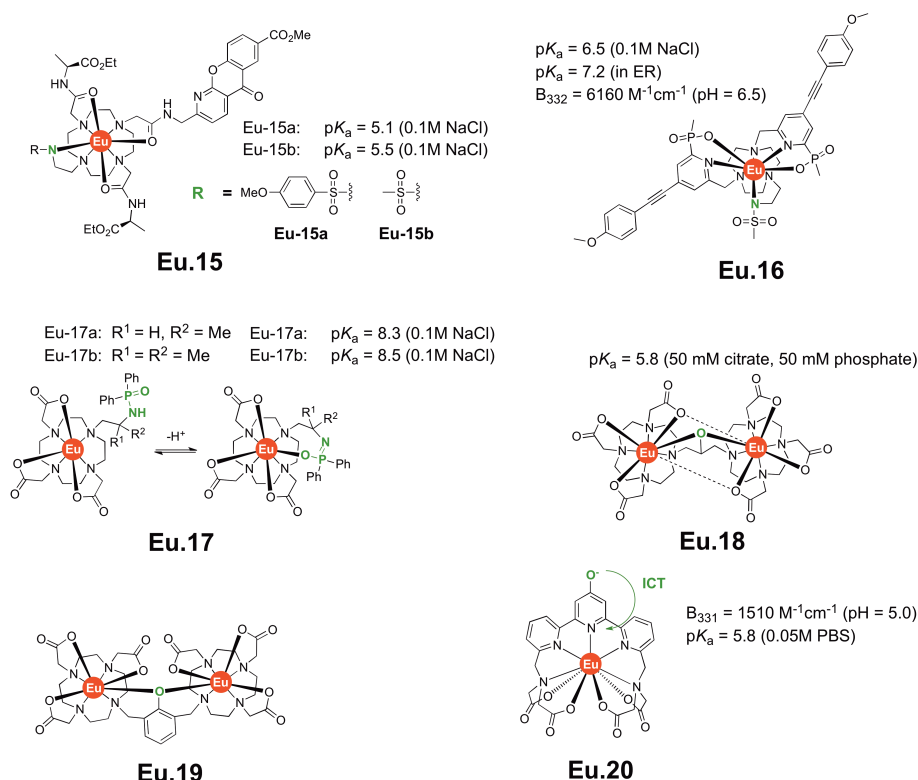


Fig. 5 Structures of selected Eu^{3+} complexes used for pH sensing.

As an example of both approaches, the europium cyclen-based complex **[Eu.15]** bearing a sulfonamide arm and an azaxanthone chromophore was devised by Parker^[23]. Reversible protonation of the sulfonamide pendant arm at nitrogen takes place (Figures 5 and 6), changing the coordination environment of europium by replacing the nitrogen of the sulfonamide with a water molecule. This change alters the emission spectral form and can be followed as a function of pH. The pK_a of the probe can be adjusted by judicious choice of the substituent in the sulfonamide arm. The use of an azaxanthone chromophore pre-determined the cellular localisation pattern – mitochondrial staining was observed over the first 4h and a predominant lysosomal staining was evident after >8h. By following the ratio between the emission intensity of terbium and analogous europium complexes, the pH value in the lysosomes of living cells was measured *in cellulo*, using spectral imaging in microscopy.

A related pH-responsive triazacyclononane europium complex **[Eu.16]** developed by the same group and bearing two arylalkynyl chromophores and a sulfonamide arm showed an endoplasmic reticulum (ER) localisation pattern^[32]. The observed pK_a of the complex in the ER (7.2) was much higher than the value observed in the case of **[Eu.15]**, due to the higher steric demand around Eu^{3+} in these 9- N_3 systems, which facilitates protonation of the sulfonamide nitrogen. Among other pH-modulating moieties a diphenylphosphineamide arm can be mentioned, whose phosphinamide oxygen can reversibly bind to Eu^{3+} as a function of pH. The Eu-D03A based complex **[Eu.17]** bearing a diphenylphosphinamide arm was reported by Lowe^[33], and showed a reversible ratiometric spectral response. Upon increasing pH, the replacement of one out of two coordinated water molecules by a phosphinamide oxygen resulted in an

increase of the luminescence intensity, with concomitant changes of the spectral profile.

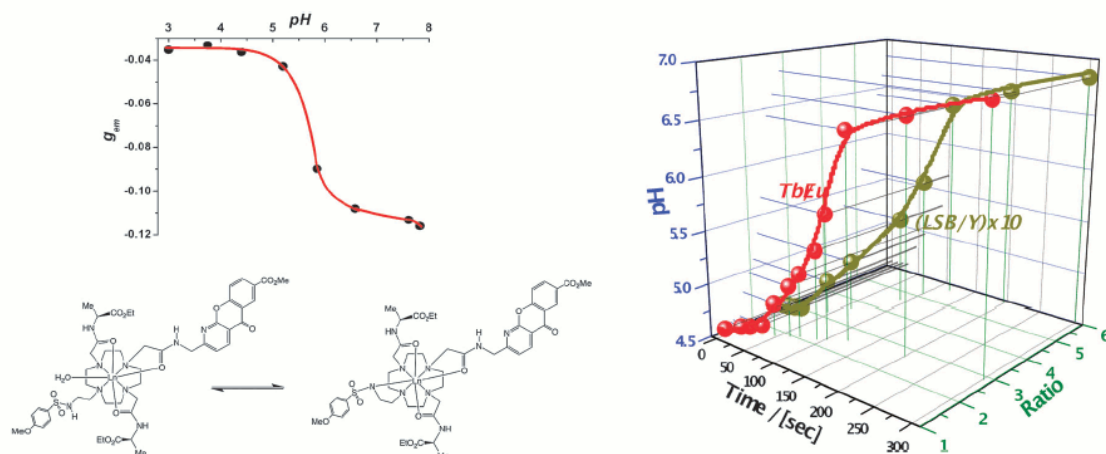


Fig. 6 The variation of the emission dissymmetry factor, g_{em} , upon varying pH of the medium for **[Eu.15a]** (left) and the change of lysosomal pH after treatment with nigericin (2 μ M) in living NIH-3T3 cells, following the Tb/Eu intensity ratio for **[Ln.15a]** (red) and blue/yellow luminescence intensity ratio of LysoSensor DND160 (right) [23]

To provide a change in the emission intensity upon protonation/deprotonation, not only the number of coordinated water molecules may be changed. Protonation itself can also introduce new O-H oscillators in close proximity to the emitting centre, as shown by Allen with the dimetallic europium complex **[Eu.18]** [34]. The complex comprised two [Eu.D03A] cores connected by an isopropyl linker that can be protonated reversibly at the bridging oxygen atom. Both the intensity and the spectral form of the complex change upon varying pH ($pK_a = 5.8$), giving rise to a ratiometric response. At the same time, although the change of the emission pattern implies the perturbation of the coordination environment of Eu^{3+} , no change in the lifetime was observed between protonated and deprotonated forms. Evidently, the deprotonated OH species is still strongly H-bonded to a second sphere water molecule.

In some cases more complex solution equilibria may occur, involving more than two species within the studied pH range. For instance, in the case of the dinuclear europium complex **[Eu.19]** [35], two [Eu.D03A] cores are bridged by a 2,6-dimethyl-4-nitrophenolate unit that can be either coordinated by two Eu^{3+} or by only one, depending on the pH of the medium, or can be protonated and bound to neither. Upon increasing pH, a rise of the emission intensity was observed, indicating that the species with a doubly coordinated hydroxyl oxygen of the bridging unit was more emissive. Since the lifetime measurements revealed that no water molecules were coordinated in either species, and no changes in the emission profile were observed at various pH values, the presence of only one emissive species was assumed (with a doubly coordinated nitrophenolate), whilst the emission from two other species was efficiently quenched by the metal-to-ligand charge transfer (MLCT) state. As a proof of concept, a mononuclear analogue was synthesized that showed no luminescence in the studied pH range, providing supporting evidence that the only emissive species in the solution of bimetallic complex within the considered pH range was the one with a doubly coordinated nitrophenolate group.

A pH-dependent luminescent response can be modulated without changing the coordination sphere of Eu^{3+} . For example, a pH-sensitive MLCT state can result in an efficient quenching of the europium emission, as demonstrated by Yuan^[36]. The Eu^{3+} complex **[Eu.20]** with a terpyridine derivative bearing four carboxylate arms showed a reversible pH-response with a pK_a of 5.8. The deprotonation of the central hydroxypyridine unit gives rise to an MLCT state that quenches Eu^{3+} luminescence at higher pH values and restores it at lower. The europium complex represents an example of a non-ratiometric pH probe, as no change in the emission pattern is observed at different pH values. However, since the emission of the terbium complex showed no pH dependence due to the absence of an MLCT state, its luminescence emission intensity can be used as a reference for the europium emission when they are used in a mixture; thereby, a red/green ratiometric probe is created.

Sensing of small molecules

Selective luminescent probing of biologically relevant small molecules is a challenging area of research, employing different mechanisms of sensing. In the case of the probes based on europium complexes, luminescent probes sensing H_2S , triplet oxygen, H_2O_2 and nitric oxide have been recently reported, (Figure 7).

The most common mechanism behind the sensing of neutral small molecules by europium complexes involves activation of an irreversible transformation of the ligand. Borbas reported^[37] a versatile platform **[Eu.21]**, in which an attached pendant arm is decorated with a protected coumarin derivative precursor. It transforms into a coumarin derivative – a well-known Eu^{3+} sensitizer – after deprotection, by reacting with an analyte. Varying the nature of the protecting group, a selective cleavage of the protective group and therefore a selective photophysical response towards a desired analyte – an enhancement of emission intensity – can be attained. For instance, the use of benzylboronic acid as a protective group creates an H_2O_2 -selective, non-ratiometric probe. However, a similar principle can yield ratiometric probes, as was demonstrated by the same group using **[Eu.22]**^[38]. This time, the coumarin derivative bearing a chemoselective cleavable group (a boronic acid, in the case of H_2O_2 signalling) in a 7-position was pre-synthesised, and therefore the probe was already emissive before adding the analyte. Since the substitution at the 7-position in coumarins can considerably perturb their frontier orbitals, cleavage of a chemoselective group at this position can change the maximum absorption wavelength of the europium complex, as revealed in the excitation spectrum. Therefore, the ratio between luminescence intensities upon excitation at these two wavelengths (before and after cleavage) can be calibrated as a function of the analyte concentration.

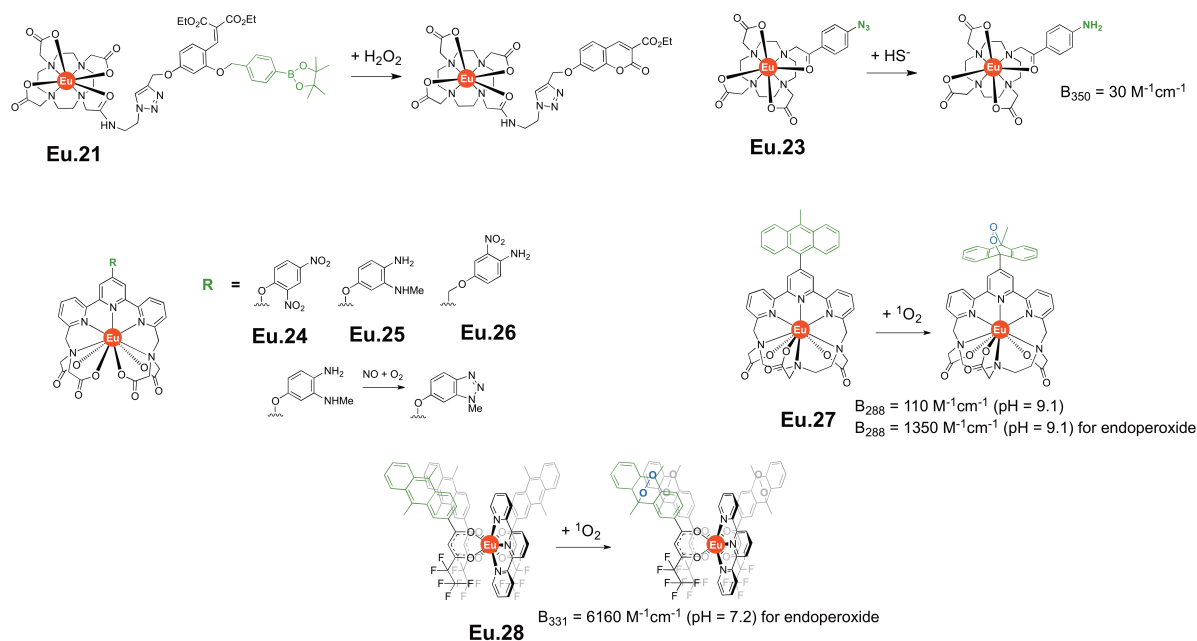


Fig. 7 Molecular structures of europium complexes for sensing of small molecules. Sensing moieties are depicted in green.

Hydrogen sulfide is naturally occurring in mammalian cells and can affect the functioning of certain ion channels; the targeting of Cl^- channels protects neurons from oxytosis.^[39] An irreversible H_2S selective [Eu.D03A]-based probe, **[Eu.23]**, has been reported by *Faulkner*^[40] and *Tuck*^[41], featuring an azido-phenacyl group, which is reduced to an amino-phenacyl moiety in the presence of H_2S . This reaction induced changes in the position of frontier orbitals, resulting in more efficient sensitization of Eu^{3+} , and hence enhancement of the total emission. A ratiometric irreversible probe for H_2S **[Eu.24]** was suggested by *Yuan*^[42], based on the terpyridine core with a 2,4-dinitrophenoxy moiety, which can be selectively cleaved in the presence of H_2S . A significant increase of the emission intensity of the terbium complex was observed, after cleaving this chemoselective group and eliminating PET, whilst the emission intensity of the europium complex was only slightly decreased. This difference in luminescence response for terbium and europium complexes allows their use as a mixture to give a ratiometric read-out. A high selectivity for H_2S over other major reactive nitrogen/oxygen species, as well as some endogenous metal ions and anions was demonstrated.

The same terpyridine platform was used to devise a closely related europium complex **[Eu.25]**^[43] for probing another essential gaseous signalling molecule, the nitric oxide radical. The terpyridine core has an attached 3-methylamino-4-aminophenoxy moiety that can be irreversibly converted into its triazole derivative by reaction with NO . The initially weak luminescence was significantly enhanced due to the decrease PET efficiency following formation of the triazole derivative. This non-ratiometric and irreversible 'dosimeter' was operational over a wide pH range (3-10), and showed good selectivity over other nitrogen/oxygen reactive species, such as H_2O_2 , NO_3^- , NO_2^- , $^1\text{O}_2$, O_2^- , ONOO^- . However, addition of the hydroxyl radical significantly reduced the emission intensity that can potentially impair the readings if NO and hydroxyl radicals are present together.

A remarkably similar complex, **[Eu.26]**, devised by the same group was employed for the detection of hypochlorous acid, which is produced by the myeloperoxidase system of phagocytes to kill a wide range of pathogens^[44]. A 4-amino-3-nitrophenoxy unit was used to selectively react with hypochlorite anions. This cleavage reaction removes the CT state and thereby drastically increased the emission intensity of the complex. Again, the operation of the probe within a wide pH range (4-10) was shown. In each of the aforementioned cases (**[Eu.25]** and **[Eu.26]**), the use of the 'cocktail' consisting of europium and terbium complexes can create a ratiometric probe, since the sensitivity of europium and terbium complexes towards the analyte was different.

Singlet oxygen ($^1\text{O}_2$) in mammalian cells can be present, as the product of different enzymatic reactions, or can be photochemically generated *in situ*, for example in photodynamic therapy (PDT) – a technique widely used for the treatment of certain diseases, including skin cancer. For instance, $^1\text{O}_2$ can readily react with a diene moiety in a [4+2] cycloaddition reaction, as in the europium complex **[Eu.27]**, reported by Yuan^[45]. A terpyridine-based platform, extensively discussed above, was used with a 10-methyl-9-anthryl moiety. This complex shows a weak luminescence due to very low-lying triplet level of the chromophore that cannot efficiently sensitize Eu^{3+} -centred emission. However, the 10-methyl-9-anthryl moiety can be readily transformed into an *endo*-peroxide upon reacting with $^1\text{O}_2$. The loss of conjugation results in a significant perturbation of the energies of frontier orbitals, which can now efficiently sensitise metal-centred emission. The irreversible enhancement of the luminescence intensity showed high selectivity over other reactive oxygen/nitrogen species. In order to improve the 'turn-on' signal ratio, the strategy employed here was developed further, resulting in a mixed-ligand complex **[Eu.28]** with three beta-diketonate ligands and one terpyridine^[46]. A beta-diketonate ligand has 9,10-dimethyl-2-anthryl moiety, whose triplet level efficiently interacts with that of terpyridine, leading to faint Eu^{3+} -centred emission of the initial complex. Again, upon reacting with $^1\text{O}_2$ the loss of conjugation results in a significant shift of the frontier orbitals, allowing efficient sensitisation of metal-centred emission. The irreversible enhancement of the luminescence intensity showed high selectivity over other reactive oxygen/nitrogen species, with a higher signal turn-on ratio and a preferential localisation in mitochondria.

Unfortunately, all the systems discussed above are irreversible 'dosimeters' that are of limited use for *in cellulo* experiments, since they cannot faithfully follow changes in the concentration of sensing analytes. The creation of analogous reversible probes remains a significant challenge, but could significantly advance progress in small molecule luminescence sensing *in cellulo*.

Nucleic acid recognition

The use of emissive europium complexes for nucleic acid recognition remains a challenge. The main issue in the creation of specific probes for nuclear DNA/RNA *in cellulo* is that their sophisticated design often inhibits permeability into the nucleus. The existing probes possess two different mechanisms to recognise DNA/RNA – either intercalating between the nucleotide bases through 'cisplatin-related' moieties, or using a synthetic DNA single strand that is matched to the

target DNA/RNA to form a triplex with DNA, or a duplex with RNA. Alternatively, peptides can be used as targeting vectors that interact selectively with RNA.

DNA recognition using intercalating agents

The use of europium complexes bearing intercalating moieties for DNA sensing has been studied by *Delangle*, who developed Ln-binding peptides [47]. To provide water solubility and an ability to integrate these molecular fragments into a peptide chain, a short amino-acid sequence was used as a core structural element to bind the Ln^{3+} ion, whilst an antenna attached to the peptide provided sensitisation of Ln^{3+} emission. The use of an antenna moiety that can intercalate between nucleotide bases can provide a 'switch-off' response, once bound to a DNA molecule, as exemplified in **[Eu.29]**[48], using a proflavine (Pfl) unit – a well-known intercalating agent. At the same time, this moiety can sensitise Eu^{3+} -centred emission, giving rise to an emissive Eu^{3+} complex. Upon addition of calf thymus DNA (CT-DNA) to the solution containing **[Eu.29]**, 45% quenching of Eu^{3+} emission was observed, whilst retaining the spectral form and lifetime of the excited state. At the same time, an absorption band corresponding to the Pfl unit was bathochromically shifted, with reduced intensity. These observations are consistent with an intercalative mechanism of binding, supported by an increase of the melting temperature of CT-DNA. The observed DNA binding constant $K_b = 10^{4.6} \text{ M}^{-1}$ is the same as the value for Pfl itself ($K_b = 10^{4.7} \text{ M}^{-1}$).

Although it was clearly demonstrated with **[Eu.29]** that a photophysical response to DNA binding can be produced, its practical application is not very promising. Firstly, antennae used as intercalating moieties generally quench the overall luminescence intensity, making it difficult to establish if the binding event actually takes place. Secondly, the lack of the internal reference signal hampers the acquisition of calibrated data. The first obstacle can be overcome by the use of 'switch-on' systems that can, for example, employ two antennae. The first one efficiently sensitises the emission of Eu^{3+} , whilst the second – capable of DNA intercalation – competes in energy transfer process with the first and quenches the metal-centred emission. However, when the second antenna intercalates between nucleotides in DNA, the emission quenching is removed and the system 'switches-on', giving rise to a non-ratiometric probe. The solution to this hurdle is to use a Eu^{3+} complex that undergoes changes in its coordination environment upon intercalation, or to use heterodinuclear lanthanide complexes, where one of the metals is sensitised by an intercalating antenna, whilst the other has an antenna that remains intact upon DNA binding.

Another solution to one of the aforementioned issues – the creation of 'switch-on' systems – has been suggested by *Patra* and is exemplified in the complexes **[Eu.30]** and **[Eu.31]**. [49] (Figure 8). The Eu^{3+} complexes bearing dipyridoquinoxaline (dpq) or dipyridophenazine (dppz) as the antenna exhibit weak luminescence in aqueous solution, due to an efficient vibrational deactivation of coordinated water molecules. Upon addition of CT-DNA, the luminescence intensity increased considerably, ascribed to intercalation of the antenna into the DNA molecule. However, the relatively low stability of these complexes in aqueous media leads to ill-defined speciation of the putative complex.

In an attempt to improve the stability and brightness of the complexes in aqueous solution, three auxiliary trifluoronaphthalenylbutanedione ligands –

widely exploited sensitisers for Eu^{3+} - were introduced into **[Eu.30]**, and the whole system, **[Eu.32]**, exhibited the usual 'turn-off' response, when CT-DNA was added.^[50] The high cytotoxicity upon UV-irradiation ($\lambda_{\text{exc}} = 365 \text{ nm}$) suggested the potential use of the complex as a ROS generator. Although the stability of the complex with respect to metal dissociation was improved compared to the parent **[Eu.30]** system, a more stable complex, **[Eu.33]**, was presented by the same group ^[51] as a 'theranostic' agent, featuring a multidentate DTPA-bisamide ligand bearing two quinoline moieties that coordinate $[\text{PtCl}_2\text{DMSO}]$ units to provide the DNA binding of the complex. Isothermal titration calorimetry measurements, following addition of CT-DNA, revealed a two-step binding process presumably involving sequential intercalation of two platinum-containing moieties into DNA. The increase of luminescence intensity upon DNA binding was partially attributed to elimination of coordinated water molecule (in line with an increased lifetime of the excited state), whilst the putative intercalation of DNA by quinoline moieties did not lead to the usually observed quenching of the Eu^{3+} emission, as the excited $^5\text{D}_0$ state of Eu is populated via an MLCT state formed by the Pt-quinoline unit. Analysis of CD-spectra showed a significant decrease in molecular ellipticity, suggesting the unwinding of the DNA helix – the mechanism responsible for an observed cytotoxicity, whereas cellular studies revealed a preferentially nuclear and 'near-nuclear' localisation pattern.

A similar principle was employed by Wong in the innovative DO3A-based complex, **[Eu.34]**, bearing an akynylpyridine chromophore with a $[\text{PtCl}_2\text{NH}_3]$ unit bound to an isonicotinamide moiety^[52]. In this work, the initial complex **[Eu.34]** is used as the prodrug that can be activated by UV- ($\lambda_{\text{exc}} = 365 \text{ nm}$) or two-photon irradiation (when exposed for 90 min), giving rise to the population of dissociative states that weaken the binding between $[\text{PtCl}_2\text{NH}_3]$ unit and the rest of the complex and leading to a controlled release of the cytotoxic drug $[\text{PtCl}_2\text{NH}_3\text{H}_2\text{O}]$. These dissociative states efficiently quenched the emission of the parent complex via quenching the singlet state of the chromophore, but after the $[\text{PtCl}_2\text{NH}_3]$ unit has been released, the quantum yield of the complex **[Eu.35]** was increased by two orders of magnitude. Notably, the incubation of **[Eu.35]** with HeLa cells did not show any uptake, whilst the complex **[Eu.34]**, after incubation and subsequent irradiation yielded an emissive species, indicating that the $[\text{PtCl}_2\text{NH}_3]$ unit promotes cellular permeability.

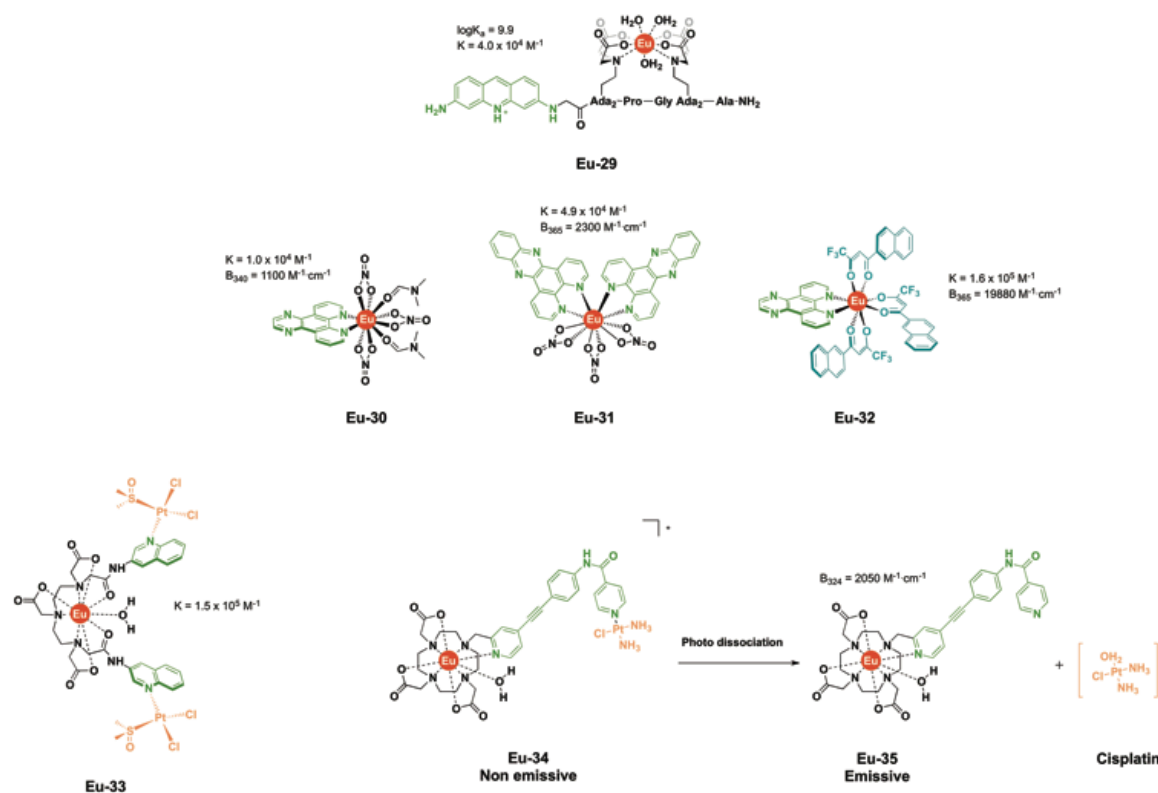


Fig. 8 Europium complexes used for DNA sensing. Antennae are depicted in green, whilst platinum moieties are depicted in yellow. Ligands depicted in cyan in **[Eu.32]** are auxiliary ligands compared to **[Eu.30]**.

DNA/RNA recognition via a template reaction

The use of pre-synthesised complementary sequences of a nucleic acid with an attached lanthanide complex can provide high selectivity towards the target nucleic acids. In a rare attempt to use such template-mediated methods to create an emissive Eu^{3+} -based probe, the complexes **[Eu.36]** and **[Eu.37]**, were reported by Abe^[53]. The complex **[Eu.36]** comprised a DTPA core (Ln-Az) attached to a methyl 2'-azido-[1,1'-biphenyl]-2-carboxylate group and an oligonucleotide (Figure 9). The second component – a triphenylphosphine carboxamide with a matched oligonucleotide (TPPc) – reduces the azide group, leading to cyclisation in the Ln-Az moiety. Once both Ln-Az and TPPc have reacted a double helix is formed bringing the Eu and sensitizer close in space, leading to sensitised Eu^{3+} emission being switched on. No Eu^{3+} -centred luminescence was detected when a mismatch was present. Therefore, Ln-Az and TPPc adopt the optimal conformation for azide reduction, only when coupled to the targeted sequence.

In another example, **[Eu.38]**, reported by Vazquez^[54], a DTPA group was coupled to a peptide sequence (Tat) bearing a phenanthroline moiety for sensing of bovine immunodeficiency virus (BIV), an RNA transactivation response element (TAR). In the absence of the targeted RNA, Eu^{3+} and the antenna are separated and relatively weak emission was observed ($\phi = 0.5 \%$). However, when the TAR RNA has been introduced by Tat, the latter adopts a β -hairpin conformation, expelling a coordinated water molecule and bringing the DTPA and phenanthroline moieties close enough to improve the efficiency of Eu^{3+} sensitisation ($\phi = 16.5 \%$).

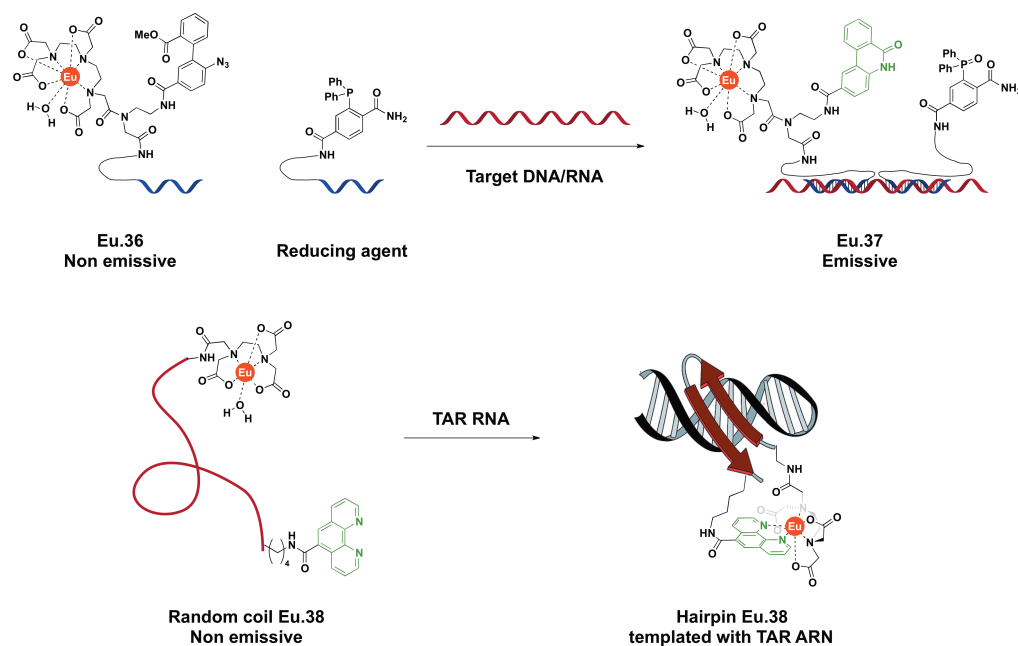


Fig. 9 Europium complexes used for nucleic acid sensing based on template reactions. Antennae are depicted in green. [54]

CPL probes for chiral species

The high sensitivity of circularly polarized luminescence (CPL) towards subtle changes in the coordination environment of chiral europium complexes makes it a very useful technique to use in addressing chiral systems. Often, significant changes in the total emission spectra do not occur, whereas the high emission dissymmetry factors, g_{em} , found in lanthanide complexes, where $g = 2(I_L - I_R) / (I_L + I_R)$ varying from 0.1 to 1.38 [55], allow CPL to be used to monitor the binding of chiral analytes. Using a racemic mixture of the Eu^{3+} complex, an induced CPL response can be monitored following interaction with a chiral analyte. Alternatively, for detection of achiral analytes, enantiopure responsive $\text{Eu}(\text{III})$ complexes can be used. Following the seminal work of Richardson, all electronic transitions in lanthanide complexes can be divided into three groups, depending on the magnitude of their dissymmetry factor. The magnetic-dipole allowed $^5\text{D}_0$ - $^7\text{F}_1$ transition of Eu^{3+} is regarded as the most sensitive for CPL studies.

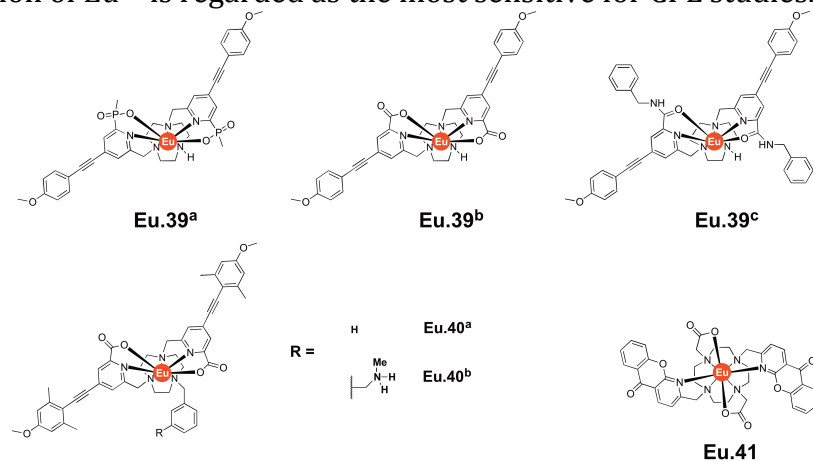


Fig. 10 Molecular structures of the europium (III) complexes used as CPL probes

There are few examples of chiral Eu^{3+} probes, wherein chiroptical signalling occurs at low complex concentrations in aqueous media. The family of achiral Eu^{3+} complexes, **[Eu.39^{a-c}]**, based on a 1,4,7-triazacyclononane (9- N_3) platform bearing alkynylpyridine moieties with different substituents – carboxylate, phosphinate and amide groups has been developed by *Parker* [56]. The binding of different oxy-anions (e. g. mandelate, lactate) by these complexes was extensively studied, allowing the enantiomeric purity of these chiral acids to be estimated. In a later paper [57], an effect of increased stabilisation of the bound analyte *via* hydrogen bonding was explored, in order to enhance analyte binding, (Figure 10 and 11). The phosphate oxygen coordinates to Eu in tandem with a directed H-bonding interaction of the bound phosphate with the protonated amine site on the ligand. For example, in **[Eu.40^{a-b}]**, a binding affinity – in the micromolar range- towards *O*-phosphono amino acids, phosphorylated and non-phosphorylated hexapeptides was found, with pronounced selectivity towards *O*-phosphono-tyrosine sites. Although the overall emission spectra did not change significantly upon binding the analyte, the CPL spectra showed high sensitivity, and the induced CPL (Figure 10) served as a spectral signature to distinguish binding at phosphorylated Tyr vs Ser/Thr residues.

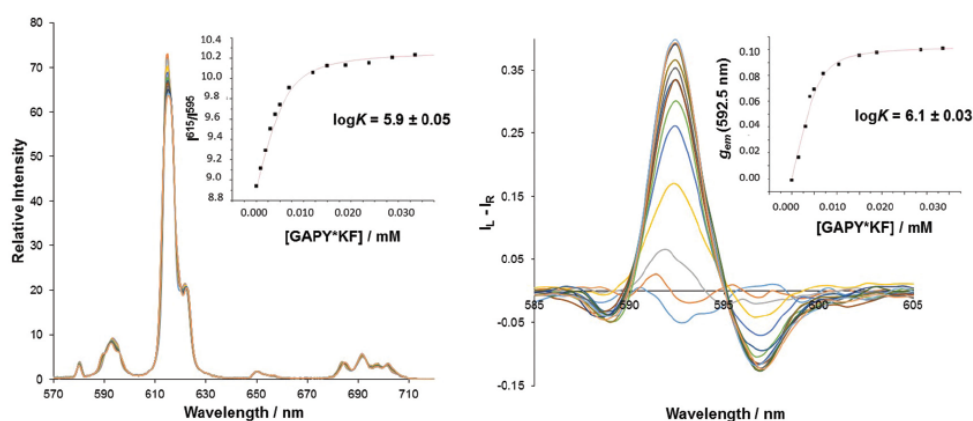


Fig. 11 The small variation of total emission intensity for **[Eu.40^b]** upon adding the *O*-phosphorylated hexapeptide contrasts with the pronounced changes in the CPL spectrum. [57]

In another system [58] developed by the same group, a racemic europium (III) complex based on a DO2A core and bearing two azaxanthone chromophores, **[Eu.41]**, was used to monitor the competitive binding of several important drugs (e.g. methadone, Imatinib and bupivacaine) that have an affinity in the micromolar range towards α_1 -AGP – an important glycoprotein found in plasma. Upon binding α_1 -AGP, the adduct **[Eu.41/ α_1 -AGP]** is formed reversibly, involving binding of a glutamate residue to Eu in concert with hydrophobic binding of one of the azaxanthone chromophores that is no longer bound to Eu. A strong “fingerprint” CPL signal was formed for the protein-bound adduct. Addition of a variety of different drugs that compete in binding to α_1 -AGP, led to dissociation of the protein-metal complex adduct with concomitant formation of the [α_1 -AGP-drug] complex. The drug binding process was therefore signalled by a decrease of the induced CPL signal intensity, allowing estimation of the drug-protein binding constant.

Two-photon excitation

Two-photon excitation microscopy has been gaining in popularity due to its intrinsic advantages over conventional confocal luminescence microscopy, e.g. the higher transparency of biological tissues in the near-IR region and the higher signal-to-noise ratio (scattered photons are too dilute to cause appreciable luminescence)^[59]. Each factor contributes to an increased penetration depth of this method (one order of magnitude deeper than in equivalent one-photon microscopy), provided that excitation does not cause too much local heating to inhibit long-lasting experiments being performed. The mechanism of two-photon excitation (Figure 12) includes a nearly simultaneous absorption of two photons, with half of the energy required for one-photon excitation, typically in near-IR region, populating a singlet excited state of the antenna. ^[60]

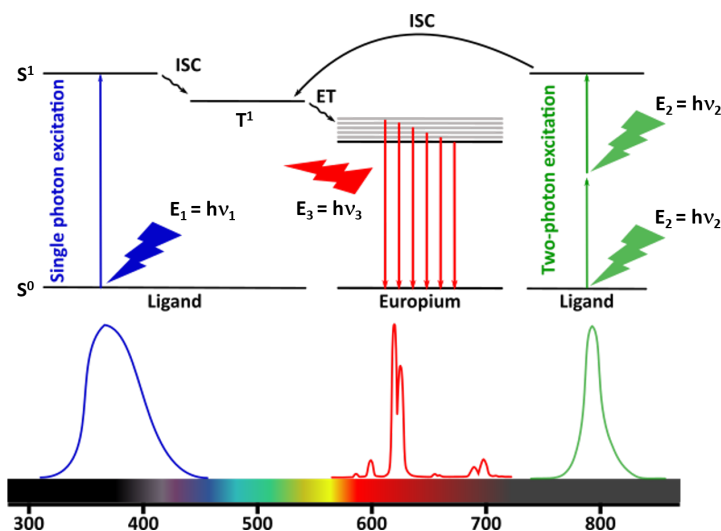


Fig. 12 Schematic mechanism of single and two-photon excitation

Various examples of europium complexes for two-photon applications have been reported so far, ^{[61],[62],[63]} including bright alkynyl-pyridine systems, functionalised with hydrophilic groups (PEG, sulfo-betaine, carboxylic acids) to ensure water solubility and cellular permeabilisation. Unfortunately, the relatively low two-photon cross-sections (<100 GM) of these systems require the use of rather high energy excitation sources that may result in lower signal intensity compared to organic dyes, such as the series of squaraine-rotaxane derivatives (up to 10,000 GM). ^[64]

In conclusion, new Eu³⁺-based systems need to be created with substantially higher two-photon cross-section values, in order that two-photon excitation probes may find wider practical application.

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